KNOWLEDGE BASED DIAGNOSIS OF INOCULUM PROPERTIES AND STERILIZATION TIME IN LACTIC ACID FERMENTATION

Terhi Siimes[1]*, Mikio Nakajima[2], Hideo Yada[2], Hajime Asama[2], Teruyuki Nagamune[2], Pekka Linko[1] and Isao Endo[2]

[1]Laboratory of Biotechnology and Food Engineering,
Helsinki University of Technology,
SF-02150 Espoo, FINLAND

[2]Chemical Engineering Laboratory,
RIKEN (The Institute of Physical and Chemical Research),
Wako, Saitama 351-01 JAPAN

* Visiting researcher from Helsinki University of Technology at RIKEN

SUMMARY

A knowledge based system has been shown to be a powerful tool for diagnosing microbial activities during a fermentation process. Knowledge about lactic acid fermentation was collected by an experimental study of *Lactobacillus casei*. The effects of the inoculum properties and sterilization time on the cultivation were expressed in a form of a fuzzy rule-based knowledge network. The system was able to detect abnormal inoculum or sterilization conditions which caused malfunctions in the cultivations.

INTRODUCTION

We have previously demonstrated the advantages of knowledge-based systems capable of handling inexact process information in diagnosing and control of fermentation processes (Asama *et al.*, 1990, Siimes, *et al.*, 1992). The system shell was initially constructed by using Smalltalk/V286 object oriented programming environment and implemented in an IBM compatible computer (Aarts *et al.*, 1989). The shell is based on the idea of using fuzzy reasoning in handling process data and knowledge, and provided a tool for representing knowledge in the form of a rule-based knowledge network (Aarts *et al.*, 1990). For the present work the shell was transferred to Smalltalk/VMac operating in a Macintosh IIci computer. While constructing the knowledge base for the diagnosis and control of lactic acid fermentation by *Lactobacillus casei* it was observed that the medium sterilization time and the characteristics of the inoculum had a marked influence on physiological activities and lactic acid production.

The first report on adverse effects of heat sterilization of lactose broth on fermentation is believed to be that of Hasseltine (1917). The first systematic study on the advantages of controlled medium sterilization is that by Pfeifer and Vojnovich (1952) who demonstrated that riboflavin production by *Ashbya gossypii* can be increased up to 8-fold if the heat damage to medium glucose is reduced. Recently Linko *et al.* (1992) demonstrated the necessity of separate sterilization of the medium components in β-galactosidase production by *Streptococcus thermophilus*. The loss of fermentable sugars during batch

sterlization can be considerable. Maillard reaction products inhibitory to micro-organisms such as hydroxymethyl furfural are formed, and filter- or HTST-sterilization is often recommended.

Few systematic studies have been reported on the effects of the size of the inoculum on the maximum lactic acid concentration and volumetric productivity. Too small of an inoculum can result in an excessively long lag phase, while a large inoculum can result in a rapid cell growth with a decreased product concentration. Lievense *et al.* (1990) observed with *L. plantarum* that during fermentation lactic acid production increases linearly with an increase in cell quantity of up to 4 g (dry weight) per liter, and defined the activity of the cell suspension as $\Delta pH \ min^{-1}g^{-1}$.

Recently Chiarini *et al.* (1992) studied the effect of varying inoculum from 1 to 10% on lactic acid production by *L. helveticus*. They observed that on unsupplemented filter-sterilized whey ultrafiltrate the highest lactic acid concentration in 24 h under controlled pH was obtained with the highest 10% inoculum. However, when the medium was supplemented with 1.5% of yeast extract the size of the inoculum had little effect on the maximum lactic acid concentration, but the peak concentration was obtained already in 11h. It appears quite clearly that the size of the inoculum can also markedly affect the results.

In the present work an object-oriented knowledge-based system was constructed for the diagnosis of faults in lactic acid fermentation by *L. casei* caused by varying sterilization conditions and inoculum properties.

MATERIALS AND METHODS

Microorganism and culture conditions

Batch cultivations were carried out with the strain *Lactobacillus casei* ATCC 27092. The culture was stored at -40°C. Seed cultures were grown on a Rogosa medium (Constantine and Hansen, 1962) at 35°C, pH 6.5 for 20 or 24 hours. The lactic acid production medium contained per litre: 25 g glucose, 25 g clarified corn-steep liquor, 1 g KH_2PO_4, 1 g K_2HPO_4, and 0.08 g $MnSO_4 \cdot 2H_2O$. The medium was sterilized using sterilization times of 10, 20, or 30 min. The size of the inoculum was varied, using amounts of 75, 80, 90, 100 and 125 ml. Experiments for collecting data concerning the standard specific rate profiles were run by using a 20 h precultivation time and 100 ml inoculum size. All of the fermentations were carried out using 2.5 dm^3 (2 dm^3 working volume) fermentors (Iwashiya, Japan) at 35°C, pH 6.5, and 150 rpm. The experimental data were stored in a specially constructed MS Excel spreadsheet at a sample-time interval of 30 minutes.

Computer system and programming environment

The knowledge based expert system employed in the present work was constructed with object-oriented Smalltalk/V Mac programming environment (Digitalk, USA). The system was implemented in the Mac IIci computer with 68020 processor, 100 MB hard disk, and 8 MB main board memory extended to 16 MB with Virtual 0'30 software. A database including the standard target data for lactic acid fermentation was also created. For on-line diagnosis the system was connected to the process control system BIOACS (Endo *et al.*, 1989) running in Fujitsu A50 as described by Siimes, *et al.*, (1992). Knowledge based diagnosis of

lactic acid fermentation was tested with off-line simulations using real experimental data according to Nakajima *et al.*, 1992.

Analyses

Biomass was measured off-line spectrophotometrically (Hitachi 100-50 uv/vis) at 660 nm. Glucose was determined off-line with Glucose B-test (Wako Pure Chemical Industries, Japan), and lactic acid with the lactic acid F-kit (Boehringer-Mannheim, Yamanouchi, Japan). The dimensionless specific rates within the range of [0,1] calculated according to Endo and Nagamune (1983) as the function of time were employed in the diagnosis. In order to smooth the experimental data 1st order delay filters with a time constant of about 6 h was used.

RESULTS AND DISCUSSION

Process knowledge

The time courses for the target profiles of biomass, glucose and lactic acid concentration were determined from batch fermentations run under the optimal conditions, and respective profiles for the specific rates were calculated and stored in the database. The standard specific rate profiles were divided into three different phases: lag-, exponential- and decreasing phase.

On the basis of the experimental data, and of other process knowledge fuzzy sets with the corresponding membership functions were defined for the process variables (Table 1.). For the precultivation time (precultTime) three fuzzy sets were defined: "short", "normal" and "long", for the inoculum size (inocSize) the fuzzy sets were: "small", "normal" and "large", and the fuzzy sets for sterilization time (sterilTime) ,were 'normal' and 'long'. Simple trapezoidal functions were used with the corner points denoted by a_1-a_4 as shown as an example in Fig. 1.

Table 1. Fuzzy sets for the fuzzy parameters.

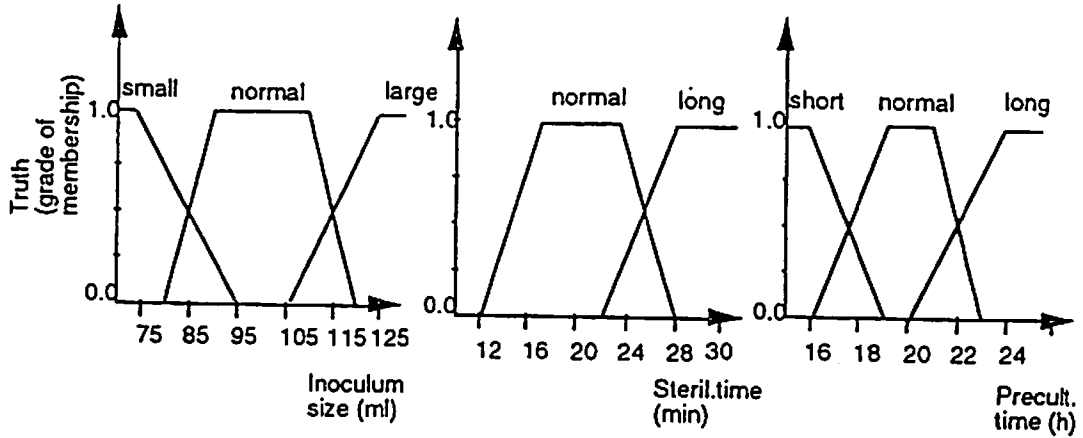|	a_1	a_2	a_3	a_4
inoculum size (ml)				
'small'	nil	nil	75	95
'normal'	80	90	110	120
'large'	105	125	nil	nil
precultivation time (h)				
'short'	nil	nil	16	19
'normal'	16	19	21	23
'long'	20	24	nil	nil
sterilization time (min)				
'normal'	12	17	23	28
'long'	22	28	nil	nil

Fig. 1. Fuzzy membership functions for inoculum size, sterilization time and precultivation time.

The main part of the process knowledge was expressed as a knowledge network constructed on the basis of *if-then* rules, describing causality of phenomena observed in the lactic acid fermentation in a graphical form using nodes and arcs (Fig. 2.). The so-called end-nodes included the faults that the system was able to detect, such as "specific substrate consumption rate is 'low'" or "product formation rate is 'low'". The original reasons to the malfunctions such as "sterilization time is 'long'" or "inoculum size is 'small'" were stored in the start-nodes. The chain of the nodes between a start-node and end-node, varying in length, consisted of and-, and or-nodes, forming a part of the *if-then* rules of the system. The intermediate nodes described facts from the cultivation such as "cell mass is 'low'".
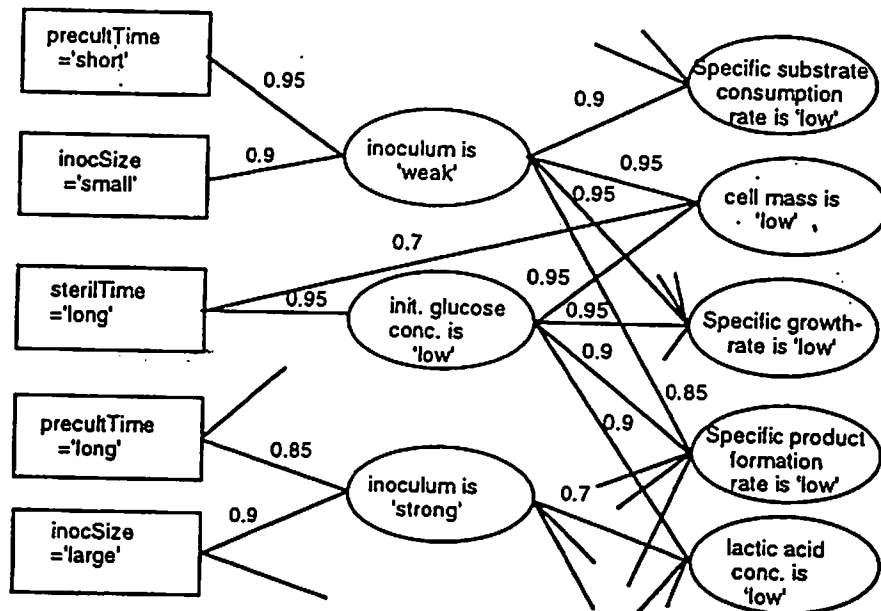

Fig. 2. Part of the knowledge network representing rules for the inoculum and substrate sterilization conditions. The text in the rectangular start- nodes on the left is written in Smalltalk syntax. The ellipsoidal end-nodes are on the right.

388

The certainty factors above the arcs represented the relative strength of the connection between the two nodes. The certainty factors were subjectively defined on the basis of several cultivations run under various conditions, considering the whole context of the knowledge network. The overall truth value for the rule chain was calculated by multiplying the fuzzy truth value of the start-node obtained from the fuzzy membership function on the basis of the respective measured value for the variable or the value of the constant parameter, with the certainty factors in the connections between the nodes, as described in Siimes et al. (1992). Fig. 2. summarizes the results in a form of a partial knowledge network, describing the the faults caused.

Knowledge based diagnosis

According to the experimental results both the precultivation time and inoculum size affected the specific rates. For example a fuzzy 'small' inoculum size and fuzzy 'short' precultivation time extended the lag phase of the growth period and decreased the specific rates. With fuzzy 'very long' precultivation times similar results were observed, because the cells were losing their viability. On the other hand, when the inoculum size was fuzzy 'large' or precultivation time fuzzy 'long', the exponential growth phase was reached earlier, and the specific growth rate was increased to higher than 'normal'. Fuzzy 'long' sterilization time caused the specific product formation rate to be low due to low initial glucose concentration. The quantity of substrate glucose decreased during an extended sterilization period, in a good agreement with White (1954).

The fault diagnosis was based on the time profiles of the biomass, glucose and lactic acid concentrations determined on-line by using a turbidity sensor and an HPLC unit connected to the BIOACS process controll system (Endo et al., 1989), and on the on-line calculation of the specific rates of cell growth, substrate consumption and product formation as described by Asama et al. (1990). The different states of microbial activity could be easily observed from the specific rate profiles. The system compared the standard data with the measured data, which had to agree to within set limits, and reported the exact current state of the cultivation to the operator (Pokkinen et al., 1992). Both the phase of the cultivation and the values of the specific rates at a certain time point were considered in diagnosis. If any difference was found, the system started the fault diagnosis process. Further, when comparing the measured specific rates with the standard values, the measured rates were reported to be either fuzzy 'low', 'high' or 'in limits'.

Fault diagnosis was realized through backward chaining to find the original causes to faults detected. For example, if the specific growth rate was found to be fuzzy 'low', the backward chaining could detect for example the following chain "specific growth rate is 'low', inoculum is 'weak', precultivation time was 'short' or inoculum size was 'small'" (Fig. 2.). All of the possible rule chains were examined by the system and the respective truth values calculated. The chain of the highest total truth value, together with the most likely root causes were reported to the operator. For example, if the specific product formation rate was found to be 'low', the system could find three possible causes to this fault according to Fig. 2. as follows:

• "Sterilization time is fuzzy 'long'" with the overall truth value of 0.43 (0.95×0.9×0.5); exact value of sterilization time was 25 min. and respective fuzzy truth of sterilTime = 'long' was 0.5 calculated from the membership function, or

• "Precultivation time is fuzzy 'short'" with the overall truth value of 0.0 (0.95×0.85×0.0); the exact value was 20 h, and respective fuzzy truth of precultTime = 'short' was 0.0, or
• "Inoculum size is fuzzy 'small'" with the overall truth value of 0.57 (0.9×0.85×0.75); exact value was 80 ml, and respective fuzzy truth of inocSize = 'small' was 0.75.

By comparing the overall truth values of each possible chain, the system reported the 'small' inoculum size to be the most likely cause to the 'low' product formation rate. This reasoning proved to be right because 'small' inoculum size lead to slower cell growth during fermentation which also decreased the product formation.

The fault diagnosis was tested running simulations with real filtered measurement data. The reasoning of the root causes of the abnormal specific rates using the knowledge base worked well. When the measured data from the cultivations based on a 'weak' or 'strong' inoculum or 'long' sterilization time was given, the system was able to detect the reasons for the abnormal conditions reliably.

ACKNOWLEDGEMENT

REFERENCES

Aarts R.J., Seppä L., Eerikäinen T., Linko P. (1989) In: Spiess WEL, Shubert, H. (eds) Engineering and food, Vol. 1, *Physical Properties and Process Control*, Elsevier Applied Science, London, pp. 909-918.

Aarts, R.J., Suviranta, A., Rauman-Aalto, P. and Linko, P. (1990) *Food Biotechnol.*

Asama, H., Flores Bustamante, Z.R., Pokkinen, M., Aarts, R.J., Takuwa, T., Nagamune, T., Endo, I. and Hirata, A. (1990) *Proc. RIKEN Symp. Bioprocess Eng.*, Feb. 2, 1990, Wako, Japan, pp. 47-49.

Chiarini, L., Mara, L. and Tabacchioni, S. (1992) *Appl. Microbiol. · Biotechnol.* 36:461-464.

Constantine E. and Hansen T. (1962) *J. Int. Dis.* 110:258-267. ·

Endo, I., Asama, H. and Nagamune, T. (1989) In: Fiechter, A. Okada, H. and Tanner, R.D. (eds.) *Bioproducts and Bioprocesses*, Springer-Verlag Berlin, pp. 337-346.

Endo I. and Nagamune T. (1983) *Ann. New York Acad. Sci.* 413:88-230.

Hasseltine, A. (1917) U.S. Public Health Reports 32.

Lievense, L.C., Van't Riet, K. and Noomen, A. (1990) *Appl. Microbiol. · Biotechnol.* 32: 669-673.

Linko, S., Enwald, S., Vahvaselkä, M. and Mäyrä-Mäkinen, A. (1992) *Ann. New York Acad. Sci.* (in press).

Nakajima, M., Siimes, T., Yada, H., Asama, H., Nagamune, T., Linko, P. and Endo, I. (1992) Proc. *ICCAFT 5/ IFAC-BIO 2 conference*, March 30- April 2, 1992, Keystone, Colorado.

Pfeifer, V. and Vojnovich, C. (1952) *Ind. Eng. Chem.* 44:1940-1946.

Pokkinen, M., Flores Bustamante, Z.R., Asama, H., Endo, I. and Linko, P. (1992) *Bioprocess Eng.* (in press).

Siimes, T., Nakajima, M., Yada, H., Asama, H., Nagamune, T., Linko, P. and Endo, I. (1992) *Appl. Microbiol. Biotechnol.* (in press).

White, J. (1954) *Yeast Technology*, Chapman & Hall, London.